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Combined Effect of Doxorubicin and Pyrogallol on Tongue Squamous Cell Carcinoma SCC-25 Cells, an in vitro Study

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Abstract

- **Background:** Oral squamous cell carcinoma (OSCC) is the pathological type of oral cancer. Chemotherapy has become increasingly effective in OSCC therapy. Of these agents, Doxorubicin (Dox) is the most efficient cytotoxic agent but is prevented by cardiac toxicity. Several experimental results demonstrated the use of a combination of low-toxic secondary metabolites, such as polyphenols, to reduce the required dose of cytotoxic agents in cancer treatment.
- **Objective:** The study aims to analyze the influence of Dox alone and its combination with pyrogallol (PG), a phenolic extract on the tongue SCC-25-cell, and to evaluate possible apoptotic pathways in the SCC-25 cells.
- **Methods:** Image morphometric analysis for the estimation of nuclear area factor (NAF) and Evaluation of caspase-3 fold change by Real-Time PCR.
- **Results:** The data recorded showed a significant decrease in the mean values of NAF of SCC-25 cells treated with different concentrations of Dox and its combination with PG. While, the combination revealed the least in the mean values of NAF when compared to control cells. Also, the combination induced apoptosis by simultaneous up-regulation of caspase-3.
- **Conclusions:** The combined use of Dox and PG may increase the effectiveness of treatment and reduce negative side effects.

Keywords

Doxorubicin, Pyrogallol, Nuclear Area Factor, Apoptosis, Real-Time PCR.

Introduction

Oral squamous cell carcinoma (OSCC) is the pathological type of oral cancer that represents the 8th most common cancer worldwide. and poses a significant threat to health, with <60 percent of patients surviving more than 5 years of age **[1]**. The oral tongue, the floor of the mouth, the buccal surface, the alveolar surface, and the hard palate can cause these tumors. The oral tongue, which is also one of the worst predicted subsites, is the most common subsite **[2]**.

Chemotherapy has become increasingly effective in OSCC therapy. Several chemotherapy drugs for cancer therapy have different modes of action, doses, and routes of administration **[3]**. Of these agents, Doxorubicin (Dox) is among the most active cytotoxic agent having efficacy in malignancies either alone or combined with other cytocidal agents. The clinical efficacy of the Dox has been prevented by cardiac toxicity. Cardiac protection during the use of Dox in cancer treatment can therefore be achieved by reducing its cumulative dose **[4]**.

Combination chemotherapy is a strategy for cancer treatment that uses multiple drugs. This approach can overcome the disadvantages of monotherapy and enhance the therapeutic effects of cancer treatment **[5]**. Several experimental results demonstrated using a combination of low-toxic secondary metabolites, such as polyphenols, to reduce the required dose of cytotoxic agents in cancer treatment **[6]**. Recent phenolic compound studies have suggested that syringic acid, tannic acid, caffeic acid, ferulic acid, coumaric acid, gallic acid, rosmarinic acid, epicatechin, and pyrogallol are present in several different proportions of honey samples **[7]**.

Pyrogallol (PG) is a simple phenol consisting of three ortho-and meta-position hydroxyl groups of the benzene ring. Humans are exposed to PG by ingestion of tea and coffee **[8]** and by gallic acid decomposition in the colon **[9]**. PG itself was shown to improve apoptosis of several types of cells, including SNU-484 gastric cancer cells **[10]**, Calu-6 human pulmonary adenocarcinoma cells **[11]**, U937 human histiocytic cells **[12]**, and endothelial cells **[13]**.

Now it is recognized that caspases are essential proteases that activate and mediate apoptotic cell death via a sequence of protein cleavage **[14]**. Caspase activation is the initial stage of apoptosis, with caspase-3 being the most main caspase executor. Caspase-3 plays a vital role in cell morphological changes and the biochemical events associated with apoptotic processes being implemented and completed **[15]**.

In this study, we investigate the potential apoptotic effects of PG/Dox combination on SCC-25 cells evaluating the cytological changes that might occur in the combination-treated cells compared to untreated control cells; analyze the nuclear morphometric changes, and evaluating caspase-3 gene expression.

Material and Methods

Reagents

PG (Sigma Aldrich-USA), with a molecular formula of $C_6 H_3 (OH)_3$ and a molecular weight of 126.11 g/mol, Dox (Sigma Aldrich- USA), the molecular formula of C27H29NO11, and molecular weight of 543.5 g/mol. The two drugs were tested for 24 hours in combination with equal doses (50:50).

Cell Culture and treatment

The human tongue SCC-25 cell line was collected from the cell culture department-VACSERA-EGYPT. SCC-25 cells were imported from the “American Type Culture Collection (ATCC)” in the form of a frozen vial with the reference number “CRL-1628”. SCC-25 cells were cultured in a minimum essential medium modified with Hank’s salts (MEM-H) supplemented

with 10% foetal bovine serum (FBS), 2mM glutamine, and sodium bicarbonate (Invitrogen, USA).

Microscopic examination

After preparation of the slide, Hematoxylin and Eosin Staining was applied by following steps:

- The fixed slides were rehydrated at low alcohol concentrations (100%, 90%, 75% then 50%) and then washed in distilled water for 5 min.
- The slides were submerged n filtered hematoxylin stain for 3 min and then washed with distilled water twice.
- The slides were soaked in filtered eosin stain for 5 seconds and then washed with distilled water.
- Dried slides were soaked in xylene, mounted with Canada balsam then coverslips were placed and left to dry.

Assessment of Hematoxylin and Eosin stained SCC-25 cells

a) Photomicrography and cytological evaluation:

Thirty microscopic fields of each group were photomicrographed at a power of 1000X (Oil immersion). This was achieved using a digital video camera (Canon, Japan) which was mounted on a light microscope (Olympus BX60, Japan). Then, images were transferred to the computer system for analysis. The selection of field was based on the existence of the highest number of apoptotic cells. The photomicrographs were assessed for the presence of morphological criteria of apoptosis.

b) Nuclear Morphometric Analysis:

The photomicrograph fields were analyzed using image analysis software (Image J, 1.27z, NIH, USA). Images have been checked for brightness and contrast automatically. Corrected images have been converted into an 8-bit grayscale type. The phase color coding was done automatically for the desired region. For selecting the nuclei of SCC-25 cells, the color threshold was adjusted. To standardize the method for all analyzed images, attempts were made to reduce the operator guided in favor of the automatic thresholding throughout this step. The surface area and circularity of the nuclei were automatically measured. The nuclear area factor has been determined using the following formula **[16]**:

Nuclear area factor = Circularity x Object area

The data were then tabulated in a Microsoft Excel sheet (Microsoft Office 2010®).

c) Statistical Analysis

The mean values of NAF of Dox and its combination with PG to the control results were statistically assessed using the Social Science Statistical Package (SPSS 16.0) window software. The statistical tests carried out included analysis of variance (ANOVA) for the comparison of the

mean of Dox and its combination with PG. Results were considered to be significant when P was <0.05. Post Hoc multiple comparisons test (Turkey HSD) was performed. Graphs representing error bars of means ± standard error were performed using SPSS software.

Estimation of caspase-3 by Real-Time PCR

Total RNA was extracted from control and treated SCC-25 cells using the Cat.no 74004 RNeasy micro kit according to the manufacturer's protocol. The concentration and integrity of the RNA were measured spectrophotometrically at a ratio of 260/280 nm. First-strand cDNA was synthesized with 1µ total RNA using the Quantitect Reverse Transcription Kit [Qiagen, Germany] following the manufacturer's instructions. These samples were subsequently frozen at a temperature of -80ºC until used for determination of the expression level of the caspase-3 gene using real-time PCR. Quantitative real-time PCR was performed on a Rotor-Gene Q cycler [Qiagen, Germany] using QuantiTect SYPR Green PCR kits [Qiagen, Germany] and forward and reverse primer for the gene.

The nucleic acid sequences of the primers were as follows:

Casp3 F: 5′-GTGGAACTGACGATGATATGGC -3′

Casp3 R: 5′-CGCAAAGTGACTGGATGAACC -3′

Compared to β-actin as a housekeeping gene

β-actin F 5’-GTGACATCCACACCCAGAGG-3’

β-actin R 5’-ACAGGATGTCAAAACTGCCC-3’

Real-time PCR mixture consisted of 25 µl 2x SYBR Green PCR Master Mix, 1.5 µl of each primer, 2 µl cDNA, and 11 µl Rnase-free water in a total volume of 50 µl.

Amplification conditions and cycle counts were a temperature of 95º C for 15 min for the initial activation, followed by 40 cycles of denaturation at 94º C for 15 sec, annealing at 60º C for 30 sec, and elongation at 72º C for 30 sec. Melting curves were performed after real-time PCR to demonstrate the specific amplification of single products of interest. A standard curve assay was performed to determine the amplification efficiency of the primers used. Relative fold changes in the expression of the target gene were accomplished using the comparative cycle threshold (2-ΔΔ CT method) with the β-actin gene as an internal control to normalize the target gene expression. ΔΔ CT is the difference between the mean Δ CT [treatment group] and the mean Δ CT [control group], where Δ CT is the difference between the mean CT gene of interest and the mean CT internal control gene in each sample. Logarithmic transformation was performed on fold change values before being statistically analyzed, using the fold change values of three replicates for each gene measured.

Results

Microscopic examination

I. Cytological evaluation

1. Control cells

Most of the SCC-25 cells showed almost rounded, hyperchromatic nuclei and the cellular outline was almost regular without evidence of any folding. Only a few cells among control cells showed criteria of apoptosis mainly confined to nuclear shrinkage and chromatin condensation (Figure 1).

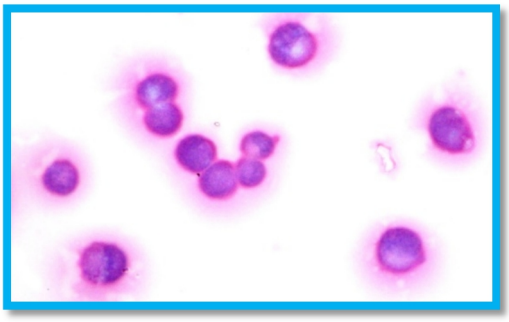
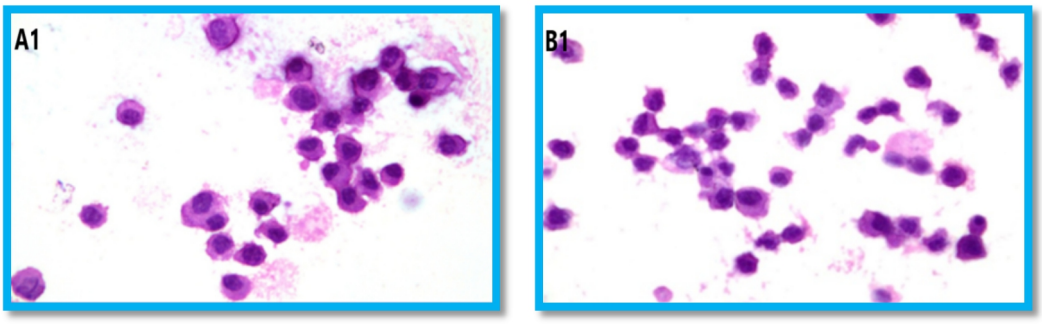


Figure (1): A photomicrograph of control SCC-25 cell 24 hours (H & E x 1000 oil).

2. Dox and Combination treated SCC-25 cells

SCC-25 cells treated with pre IC50, IC50, and post IC50 concentrations of Dox and its combination with PG for 24 hours showed apoptotic morphological changes, necrotic features, and secondary necrosis. The necrotic criteria and secondary necrosis were more dominant in post IC50 concentration of Dox such as swollen necrotic cell with clumping of heterochromatin admixed with euchromatin and necrotic cell. While, the apoptotic morphological changes such as nuclear shrinkage, nuclear fragmentation, irregularities in the nuclear membrane, peripheral chromatin condensation, blebbing of the cell membrane, and apoptotic bodies became more obvious at IC50 and post IC50 concentration of the combination of Dox with PG (figure 2).



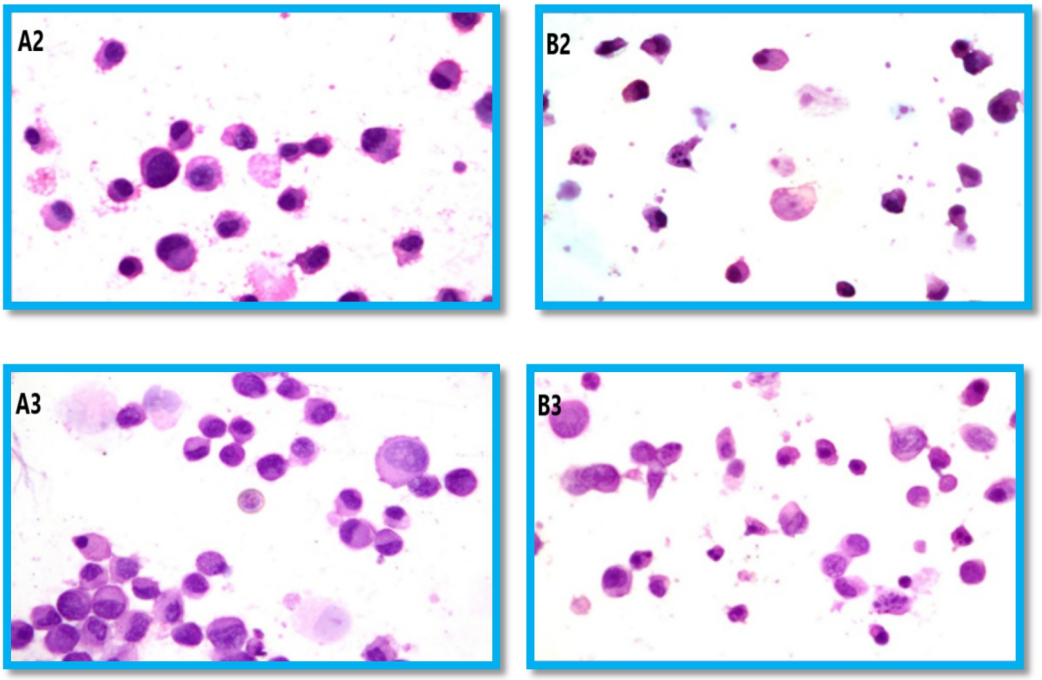


Figure (2): A photomicrograph of SCC-25 cell 24 hours post treatment with PreIC50 (A1), IC50 (A2) and Post IC50 (A3) concentration of Dox; PreIC50 (B1), IC50 (B2) and Post IC50 (B3) concentration of the combination (H & E x 1000 oil).

II. Morphometric analysis

a) Nuclear area factor (NAF)

The mean NAF values for SCC-25 cells treated with different concentrations of Dox alone and its combination with PG for 24 hours are shown in Table (1). The data recorded showed a decrease in the mean values of NAF of SCC-25 cells treated with different concentrations of Dox when compared to control cells. While, SCC-25 cells treated with different concentrations of the combination revealed more decrease in the mean values of NAF when compared to control cells (Figure 3 a, b). Over different concentrations, there was a decrease in the mean values of NAF with increasing concentration.

Table (1): Descriptive statistics for the mean values of NAF of control and SCC-25 cells treated with different concentrations of Dox and the combination after 24 hours.

	Conc. ug/ml	NAF
SCC25 treated with Dox	Pre-IC50	4212.20
	IC50	4032.24
	Post-IC50	3677.83
SCC25 treated with the combination	Pre-IC50	3935.60
	IC50	1845.64
	Post-IC50	1674.91
control		9579.82

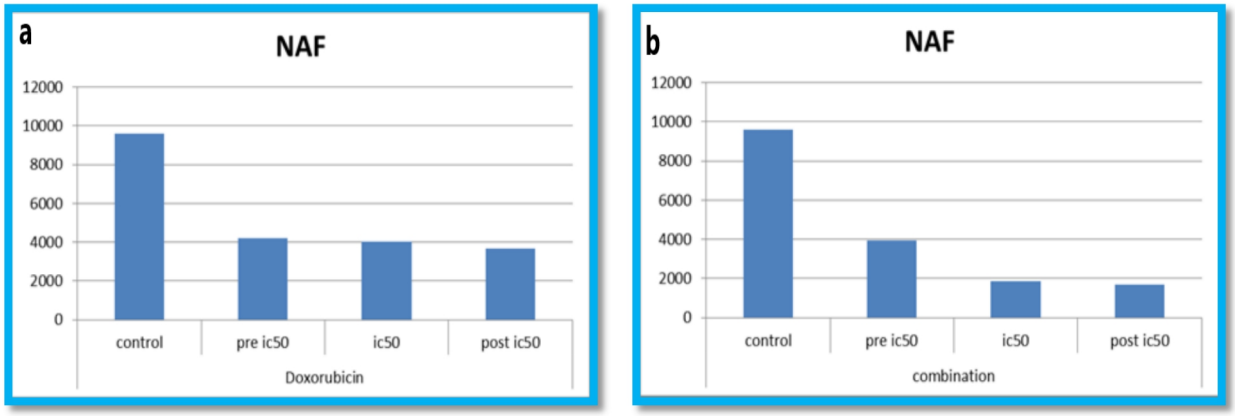


Figure (3): A histogram representing the mean values of NAF of control and SCC-25 cells treated by different concentrations of Dox (a) and the combination (b).

b) Statistical Results

ANOVA test revealed a statistically high significant difference among mean values of NAF of SCC-25 cells treated with different concentrations of Dox alone and Dox with PG and the control cells (P-value < 0.000) (Table 2, 4)

Post Hoc multiple comparison test (Bonferroni) revealed a statistically insignificant difference among mean values of NAF of SCC-25 cells treated with different concentrations of Dox alone and Dox with PG to each other. But there was a statistically significant difference among mean values of NAF of SCC-25 cells treated with different Dox and its combination with PG concentrations when compared to the mean values of control cells after 24 hours (Table 3, 5).

Table (2): ANOVA test for the mean values of NAF of different Dox concentrations and control cells 24 hours post treatment.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	237159714.845	3	79053238.282	17.538	0.000
Within Groups	162269887.540	36	4507496.876		
Total	399429602.385	39			

Table (3): Post Hoc multiple comparisons test (Bonferroni) for comparison of mean difference NAF ± standard error for different Dox concentrations and control cells 24 hours post treatment.

(I)group	Mean Difference	Std. Error	Sig.	95% Confidence Interval	
				Lower	Upper

(J) group			(I-J)			Bound	Bound
	Control	Pre IC50	5367.61710*	949.47321	0.000	2716.7145	8018.5197
		IC50	5547.57190*	949.47321	0.000	2896.6693	8198.4745
		Post IC50	5901.98870*	949.47321	0.000	3251.0861	8552.8913
	Pre IC50	Control	-5367.61710-*	949.47321	0.000	-8018.5197	-2716.7145
		IC50	179.95480	949.47321	1.000	-2470.9478	2830.8574
		Post IC50	534.37160	949.47321	1.000	-2116.5310	3185.2742
	IC50	Control	-5547.57190-*	949.47321	0.000	-8198.4745	-2896.6693
		Pre IC50	-179.95480	949.47321	1.000	-2830.8574	2470.9478
		Post IC50	354.41680	949.47321	1.000	-2296.4858	3005.3194
	Post IC50	Control	-5901.98870-*	949.47321	0.000	-8552.8913	-3251.0861
		Pre IC50	-534.37160	949.47321	1.000	-3185.2742	2116.5310
		IC50	-354.41680	949.47321	1.000	-3005.3194	2296.4858

*. The mean difference is significant at the 0.05 level.

Table (4): ANOVA test for the mean values of NAF of different concentrations of the combination and control cells 24 hours post treatment.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	402326672.839	3	134108890.946	34.417	0.000
Within Groups	136380267.951	35	3896579.084		
Total	538706940.791	38			

Table (5): Post Hoc multiple comparisons test (Bonferroni) for comparison of mean difference NAF ± standard error for different concentrations of the combination and control cells 24 hours post treatment.

(I)group (J) group			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
	Control	Pre IC50	5644.21680*	882.78866	0.000	3175.4059	8113.0277
		IC50	7734.17980*	882.78866	0.000	5265.3689	10202.9907
		Post IC50	7904.91002*	906.97913	0.000	5368.4479	10441.3721

	Pre IC50	**Control**	-5644.21680-*	882.78866	0.000	-8113.0277	-3175.4059
		IC50	2089.96300	882.78866	0.141	-378.8479	4558.7739
		Post IC50	2260.69322	906.97913	0.105	-275.7689	4797.1553
	IC50	**Control**	-7734.17980-*	882.78866	0.000	-10202.9907	-5265.3689
		Pre IC50	-2089.96300	882.78866	0.141	-4558.7739	378.8479
		Post IC50	170.73022	906.97913	1.000	-2365.7319	2707.1923
	Post IC50	**Control**	-7904.91002-*	906.97913	0.000	-10441.3721	-5368.4479
		Pre IC50	-2260.69322	906.97913	0.105	-4797.1553	275.7689
		IC50	-170.73022	906.97913	1.000	-2707.1923	2365.7319

*. The mean difference is significant at the 0.05 level.

PCR (Caspase-3 fold change)

Treatment of SCC-25 cells with different concentrations of Dox and Dox/PG combination causes significant up-regulation of Caspase-3 gene fold change compared to control cells.

It was found that Post IC_{50} concentration of Doxorubicin treated SCC-25 cells illustrating the highest value of Caspase-3 gene fold change (Table 6) (Figure 4).

Table (6): Caspase-3 gene fold change using RT-PCR in different concentrations of Dox and the combination-treated SCC-25 cells compared to control cells.

Sample data	Conc. ug/ml	Casp3 FLD
SCC25 treated with doxorubicin	pre-IC50	12.85
	IC50	25.80
	post-ic50	28.71
SCC25 treated with the combination	pre-IC50	7.68
	IC50	13.95
	post-ic50	19.58
control		1

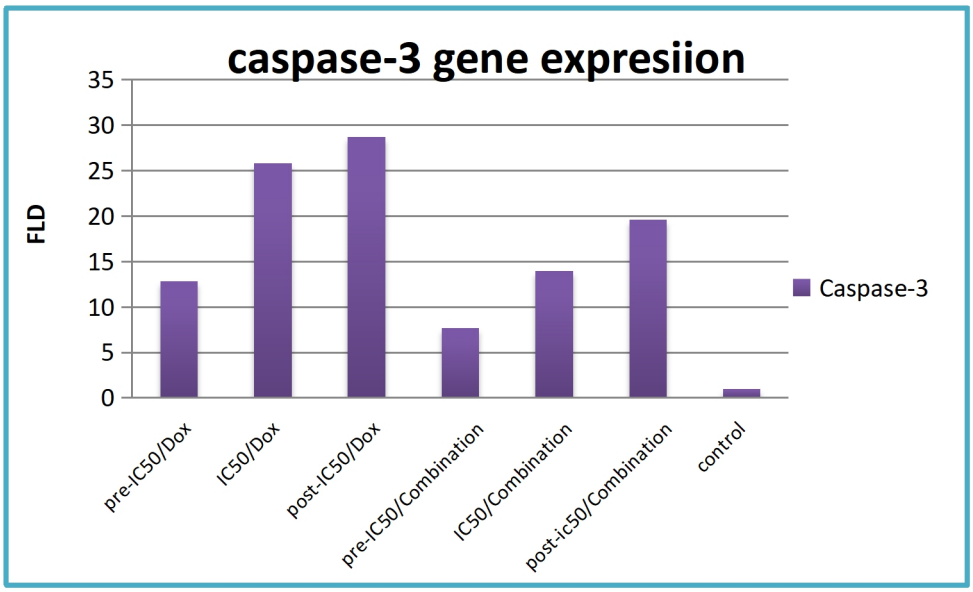


Figure (4): Evaluation of caspase-3 gene expression on different concentrations of Dox and the combination-treated SCC-25 cells compared to control cells.

Discussion:

Dox is an effective chemotherapeutic agent that is utilized used in various types of cancers **[17]**, but its clinical use is often restricted by the generation of cardiotoxicity **[18]**. Hence, combination therapy has known as an essential strategy for a better long-term prognosis with reduced side effects in cancer therapy **[19]**.

As shown in this study, Dox and the combination showed apoptotic morphological changes, necrotic features, and secondary necrosis. These combination-treated cells demonstrated that the number of necrotic cells is less than in Dox treated cells. Several in vitro and in vivo results have recorded that combining natural polyphenols with chemotherapy can enhance the effectiveness of anticancer drugs and decrease side effects **[20, 21]**

There are several biochemical and image-based essays for apoptosis that differ in complexity, specificity, and cost. It has previously shown that the NAF can be an early and sensitive indicator of cell morphological changes occurring during apoptosis because it is based on two important morphologic parameters of apoptosis **[22]**. The NAF was calculated (surface area x circularity) by using Image J, 1.27z **[16]**.

The NAF of SCC-25 cells treated by different concentrations of Dox and Dox/PG combination for 24 hours was compared as well as with NAF of control untreated cells. Where decreasing in NAF means that there were apoptotic changes. ANOVA test revealed that the mean values of NAF of SCC-25 cells treated by different concentrations of Dox and Dox/PG combination decreased compared to control untreated cells. These results supported the appearance of apoptotic changes.

This decrease is encouraged in the cytological examination which showed an increase in the apoptotic morphological changes at different concentrations of Dox and Dox/PG combination.

Furthermore, the lowest reduction in the mean value of NAF occurred at post IC50 concentration of the combination when compared to the control untreated cells. Thus, at that concentration the essential role in cell death is apoptosis.

To confirm these results caspase-3 was evaluated using the RealTime PCR technique after individual treatment of SCC-25 cells with Dox and Dox/PG combination (Pre Ic50, Ic50, and Post Ic50 at 24h). In the present study, it was observed that the expression of caspase-3 increased in SCC-25 treated cells with different concentrations of Dox and PG/Dox combination when compared to control non-treated cells, and increased with increasing concentration from pre IC_{50} to post IC_{50}.

Caspase-3 is a key caspase executioner that is cleaved and activated by both caspase-8 and caspase-9 initiator caspase **[23]**. Active caspase-3 degrades multiple cellular proteins and causes morphological changes and DNA fragmentation in cells during apoptosis **[24]**. The current results concerning Dox are in line with **Guerriero *et al.* [25]** who showed that the mRNA expression of caspase-3 genes increased significantly after treatment for 48 h with Dox in the MCF-7 breast cancer cell line. Additionally, the present findings are consistent with **Abdel-Hamid *et al.* [26]** who showed that the treatment of OSCC CAL-27 cells with Dox for 24h resulted in a significant increase in caspase-3 up to 2.72-folds of the control level.

Different studies also have indicated that Dox-induced apoptosis is associated with two distinct apoptotic pathways, i.e., the extrinsic and mitochondrial or intrinsic pathways **[27]**. The mitochondrial or intrinsic pathway is the major mechanism of Dox-induced apoptosis, in which the central process involves the change of permeability of the outer mitochondrial membrane with the subsequent release of several pro-apoptotic factors into the cytosol **[28]**. Furthermore, Dox causes apoptosis of bone marrow-derived mesenchymal stem cells (BMSCs) through ROS increase and depolarization of mitochondrial membrane potential, as well as the activation of p38, p53, Bax, and caspase-3 genes, which consequently trigger apoptosis and dysfunction of cells **[29]**.

To our knowledge, in English literature, no research was conducted to investigate the effect of the combination of Dox and PG concerning caspase-3 expression as this the first time that the combination has been prepared. In turn, the current results are supported by researchers used a combination of other polyphenols with Dox.

Mahbub *et al.* [30] demonstrated that Dox when used in combination with quercetin produced a synergistic increase in caspase-3 activity in all leukemia cell lines. Moreover, **Guerriero *et al.* [25]** indicated that in MCF-7 cancer breast cell line Dox + PEFSO induce an apoptotic intrinsic pathway by p53, Bax p38, and caspase-3 activation as well as an apoptotic extrinsic pathway by caspase-8 activation.

Conclusions:

Based on our results, the combined use of PG, and the conventional chemotherapy drug, Dox could be used to reduce the effective dose of Dox and therefore, most likely its side-effects.

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